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A molecular framework for the phylogeny of the Pseudocerotidae (Platyhelminthes, Polycladida)

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Abstract

Systematic relationships within the cotylean family Pseudocerotidae were examined using nucleotide sequences of the D3 expansion segment of the 28S rDNA gene. A previously suggested separation of *Pseudoceros and Pseudobiceros* based on the number of male reproductive systems was confirmed. Regardless of the algorithm employed, *Pseudoceros* always formed a monophyletic clade. *Pseudobiceros* appeared to be paraphyletic; however, a constrained maximum parsimony tree was not significantly longer (2 steps, $\alpha = 0.05$). Additionally, the genera *Maiazoon, Phrikoceros* and *Tytthosoceros* were validated as taxonomic entities, and their relationships to other genera within the family were determined. Molecular data also supported species separations based on colour patterns. An intraspecific genetic distance of 1.14% was found for *Pseudoceros bifurcus,* whereas the intrageneric distance was 3.58%. Genetic distances among genera varied, with the closest distance being 2.048% between *Pseudobiceros* and *Maiazoon,* and the largest distance (8.345%) between *Pseudoceros* and *Tytthosoceros.*

Introduction

Based on the character 'presence/absence of a cotyl or sucker', Lang (1888) divided the order Polycladida into two suborders, the Cotylea and the Acotylea. The systematics of polyclads was reviewed simultaneously by Faubel (1983, 1984) and Prudhoe (1985). Unfortunately, these reviews resulted in two non-concordant systematic schemes. Prudhoe (1985, 1989) following Hyman (1955a,b,c; 1959a,b), maintained that many species, especially within the colourful Pseudocerotidae, could be diagnosed solely on the basis of colour patterns. Faubel (1984) disagreed and stated that, as is common for other turbellarian flatworms, species should be separated on details of their reproductive structures. Inherent problems surfaced because both reviews relied strongly on the past literature and type material was not located or re-examined. To date, no one system is reliably used and the systematics of these turbellarians is accordingly, in a state of confusion (Newman & Cannon, 1994a).

The cotyleans, with 10–16 families (depending on author), are prominent and colourful members of reef communities (Cannon, 1986). The family Pseudocerotidae is the largest and most diverse within the Cotylea. To date, there are approximately 13 genera in the family with an estimated 500 or more species worldwide (Faubel, 1984; Newman & Cannon, 1994a,b, 1996a,b; 1997, 1998).Until Faubel's (1984) revision, *Pseudoceros* with about 75% of the named species in the family, represented the largest genus. Based on the character 'double male reproductive system', Faubel (1984) separated the genus *Pseudobiceros* from *Pseudoceros*.

However, male and female reproductive systems show a surprising homogeneity (Newman & Cannon, 1994a, 1995, 1998a,b), thus, separating species within each genus continued to be difficult. Both genera reproduce via random hypodermic insemination through the body wall (Newman & Cannon, 1994a; Michiels & Newman, 1998). Such a reproductive mode provides a convincing explanation for the re-

lative reproductive homogeneity in these flatworms. Certainly, a reproductive behaviour involving random deposition of sperm through the body wall is unlikely to generate elaborate morphological copulatory isolating mechanisms (Newman & Cannon, 1994a).

Newman & Cannon (1994a, 1995) were able to differentiate 64 new pseudocerotid species based on colour patterns. Unlike earlier descriptions, these authors examined live material and their studies were greatly aided by a new fixation technique that allowed for the preservation of pattern colour (Newman & Cannon, 1995). Furthermore, Newman & Cannon (1994a) showed that individuals of like patterns copulated simultaneously, thus mitigating against any argument of colour pattern polymorphisms within species. Additional support for species distinctiveness is seen in differences between size at maturity and in habitat (Newman & Cannon, 1994a) which indicates that species separated on colour pattern are reliable biological entities. Thus, while genera can be separated on the basis of the male reproductive system, species distinctions within a genus can rely on colour patterns.

Based on the shape of pseudotentacles, pharynx and reproductive anatomy, Newman & Cannon (1996a,b) erected four new pseudocerotid genera, *Bulaceros, Maiazoon, Phrikoceros* and *Tytthosoceros* (Table 1). As with *Pseudoceros,* the genera *Phrikoceros* and *Tytthosoceros* possess one male pore but they can be separated from *Pseudoceros* on morphological differences such as the shape of their pharynx and pseudotentacles, and the arrangement of their eyes. Only *Maiazoon* is similar to *Pseudobiceros* in possessing two male reproductive systems, simple folds of the pharynx, and deep marginal ruffling. However, it is separated from that genus by having three to five female antra. *Phrikoceros*, on the other hand, shares a single male reproductive system with *Pseudoceros* and is distinguished from that genus by folded pseudotentacles, deep marginal ruffles, and clustered dorsal and ventral pseudotentacular eyes (Newman & Cannon, 1996). To date, no independent validation of these genera has been made.

Molecular phylogenetic studies have become the standard for providing an independent test of existing morphology-based phylogenies (Halanych et al., 1995; Winnepenninckx et al., 1995; Giribet et al., 1996; Aguinaldo et al., 1997; Carranza et al., 1997; Balavoine, 1998; Adoutte et al., 2000 and references therein). In a first attempt to evaluate the usefulness of nucleotide sequence data in resolving pseudocerotid relationships, Goggin & Newman (1996) sequenced about 400 base pairs of the internal transcribed spacer (ITS1) region of the rRNA in three species of *Pseudoceros.* These authors found sufficient variation to unequivocally discriminate among the three species.

We therefore wanted to evaluate the usefulness of the D3 expansion segment of the 28S rDNA gene for the phylogenetic resolution of pseudocerotid flatworms. This segment has previously been shown to be of phylogenetic value in resolving relationships at various taxonomic levels, ranging from species to class (Litvaitis et al., 1994, 1996, 2000; Nunn et al., 1996). Our specific objectives were (1) to determine if separating *Pseudoceros* and *Pseudobiceros* based on the number of male reproductive systems is confirmed by molecular data, (2) to determine if species separated according to the colour pattern grouping system established by Newman & Cannon (1994a) are valid entities, and (3) to provide a first test of the validity of the genera *Maiazoon*, *Phrikoceros* and *Tytthosoceros.*

Materials and methods

Cotylean flatworms were hand collected from the Great Barrier Reef (GBR), Australia; Papua New Guinea and Dominica, West Indies (Table 2). DNA was extracted according to Litvaitis et al. (1994, 1996), and amplified using primers designed to conserved regions around the D3 expansion segment of the gene coding for 28S rDNA (for primer sequences, see Litvaitis et al., 1994). Amplified products were gel purified, and 4–5 μl of each sample were used in a cycle-sequencing reaction (protocol according to ABI Inc.). Fragments were separated on a 6% denaturing polyacrylamide gel, and sequences were determined using an automated sequencer (ABI 377). Initial editing of sequences was done via the SeqEd program (version 1.0.1; ABI Inc.). Although the products were only about 350 base pairs long, both strands were sequenced to assure accuracy.

Sequences were aligned by the CLUSTAL method, using MegAlign (DNA*) with further improvements of the alignment by eye. An initial neighbor joining tree (NJ) was produced (PAUP*; Swofford, 1999) using two specimens of acotyleans and the macrostomid *Microstomum papillosum* as an outgroup. Macrostomids have been shown to represent the immediate sister group of polyclads (Carranza et al., 1997; Litvaitis & Rohde, 1999). To correct for multiple substitutions, data were log/Det transformed for NJ-

Table 1. Summary of generic diagnostic characters for selected Pseudocerotidae (Faubel, 1984; Prudhoe, 1985; Cannon, 1986; Newman and Cannon, 1994a, 1996a). Note only genera used in this study are listed

Character	*Acanthozoon*	*Pseudoceros*	*Pseudobiceros*	*Maiazoon*	*Phrikoceros*	*Thysanozoon*	*Tytthosoceros*
Body shape	raised medially	flat	raised medially	raised medially	raised medially	raised medially	raised medially
Dorsal surface	papillate	smooth	smooth	smooth	smooth	papillate	smooth
Pseudotentacle shape	[a]ear-like	simple	ear-like or square	square	square	[a]ear-like	ear-like
Pharyngeal folds	[a]complex	complex	simple	simple	simple	[a]simple	simple
Cerebral eye clusters	[a]4 clusters	anterior lines	4 clusters	4 clusters	4 clusters	[a]4 clusters	scattered
Number of female pores	1	1	1	3–5	1	1	1
Number of male pores	1	1	2	2	1	2	1

[a]Newman, unpublished data.

trees. Reliability of internal nodes was ascertained by 2000 bootstrap replications. A maximum parsimony (MP) analysis using heuristic search was conducted with random sequence addition and tree bisection-reconnection branch swapping (PAUP*; Swofford, 1999). An alternative MP hypothesis was evaluated where *Pseudobiceros* was constrained into a monophyletic clade. Using the non-parametric ranked sign test of Templeton (Larson, 1994) at $\alpha = 0.05$, it was shown that the constrained tree was not significantly longer (2 additional steps). Alternative longer trees that provided a better concordance with morphological characters are favored as long as they are not statistically different from the MP tree (Litvaitis & Rohde, 1999, Litvaitis et al., 2000). We therefore used the constrained MP tree in our analysis.

Results and discussion

Regardless of the algorithm employed, the genus *Pseudoceros* always formed a distinct clade (Figs 1 and 2). The coherence of the genus is further supported by morphological characters associated with the eyes, pseudotentacles, pharynx and by details of the reproductive system (Newman & Cannon, 1994a). Within the genus, three specimens of *Pseudoceros bifurcus* formed a clade whose sister group included two

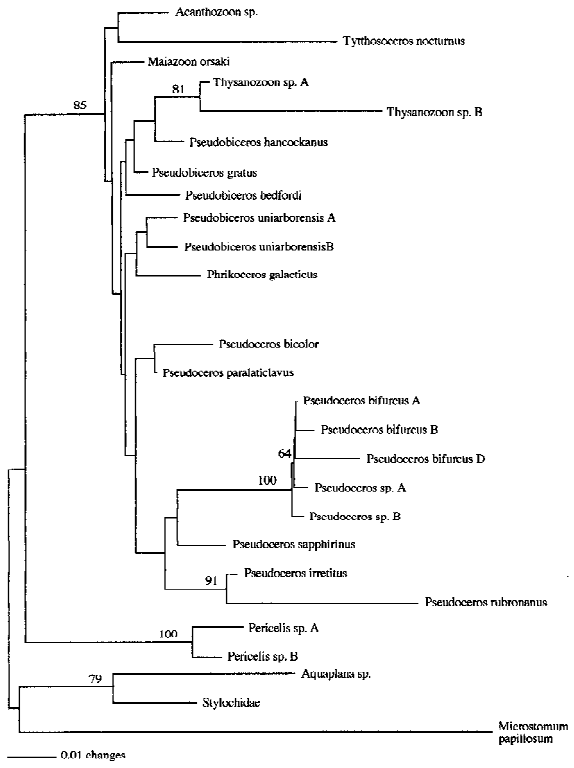

Figure 1. Neighbor-joining tree of 25 polyclad specimens, using partial sequences of the 28S rDNA gene (D3 expansion segment). Numbers at nodes are percentages of 2000 bootstrap replications; only values above 50% are reported.

Table 2. Species and collection localities

Taxon	Collection locality
Pseudoceros	
bicolor	Dominica, West Indies
bifurcus A, B	North Heron Island, GBR, Queensland, Australia
bifurcus D	Blue Pools, North Heron Island, GBR, Queensland, Australia
irretitus	North Heron Island, GBR, Queensland, Australia
paralaticlavus	North Heron Island, GBR, Queensland, Australia
rubronanus	North Heron Island, GBR, Queensland, Australia
sapphirinus	North Heron Island, GBR, Queensland, Australia
unident. sp. A and B	Point Cartwright, Mooloolabe, SE Queensland, Australia
Pseudobiceros	
bedfordi	Blue Pools, North Heron Island, GBR, Queensland, Australia
gratus	North Heron Island, GBR, Queensland, Australia
hancockanus	Heron Island, GBR, Queensland, Australia
uniarborensis A	Madang, Papua New Guinea
uniarborensis B	North Heron Island, GBR, Queensland, Australia
Acanthozoon	
unidentified sp.	Madang, Papua New Guinea
Maiazoon	
orsaki	Madang, Papua New Guinea
Phrikoceros	
galacticus	Heron Island, GBR, Queensland, Australia
Thysanozoon	
unidentified sp. A and B	Madang, Papua New Guinea
Tytthosoceros	
nocturnus	off Heron Island, GBR, Queensland, Australia
Pericelis	
unidentified sp. A	Madang, Papua New Guinea
unidentified sp. B	Lizard Island, GBR, Queensland, Australia

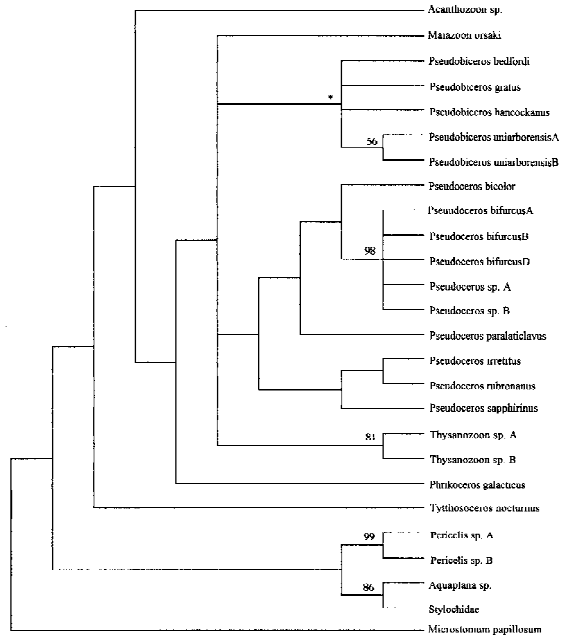

Figure 2. Consensus MP tree of 25 polyclad specimens, using partial sequences of the 28S rDNA gene (D3 expansion segment). Numbers at nodes are percentages of 2000 bootstrap replications; only values above 50% are reported. Note, *Pseudobiceros* was constrained as a monophyletic group (*). (Tree length 184; CI = 0.723; RI = 0.702; RC = 0.507).

unidentified species of *Pseudoceros* (A and B). The average genetic distance among the three specimens of *P. bifurcus* was 1.14%. Using this value as a measure of intraspecific variation, we compared genetic distances among different *Pseudoceros* species that had been separated based on colour patterns. A genetic distance value of 0.609% between *Pseudoceros* sp. A and *Pseudoceros* sp. B led us to conclude that the two specimens were very likely the same species. Both specimens were cream-coloured. Although one was surrounded by a continuous purple margin, whereas the margin of the second specimen was discontinuous and slightly more blue in colour, these differences fall well within the range of variation observed for other pseudocerotid colour patterns.

A comparison of genetic distances of the two unidentified *Pseudoceros* species with *P. bifurcus* D (1.651% and 1.636%, respectively) showed that the two may be closely related to *P. bifurcus*, a conclusion that was further supported by 100% bootstrap replications (Fig. 1). The average genetic distance within the genus *Pseudoceros* was 3.58%, indicating that species separations based on colour patterns are valid

Table 3. LogDet/paralinear distance matrix of pseudocerotid genera. Values for *Thysanozoon, Pseudoceros* and *Pseudobiceros,* represent intrageneric averages

Acanthozoon	*Maiazoon*	*Phrikoceros*	*Pseudobiceros*	*Pseudoceros*	*Thysanozoon*	*Tytthosoceros*	
Acanthozoon	—						
Maiazoon	0.02052	—					
Phrikoceros	0.03483	0.03044	—				
Pseudobiceros	0.02630	0.02048	0.02354	—			
Pseudoceros	0.05162	0.04632	0.04707	0.04101	—		
Thysanozoon	0.04997	0.05856	0.05462	0.04281	0.06951	—	
Tytthosoceros	0.05584	0.06932	0.06106	0.06369	0.08345	0.08220	—

and allow for a reliable discrimination of individual species.

The genus *Pseudobiceros* proved to be a more heterogeneous group (Fig. 1). Unconstrained analyses resulted in a paraphyletic genus with most of the examined species grouping with members of *Thysanozoon* and two specimens of *Pseudobiceros uniarborensis* clustering with *Phrikoceros galacticus.* A close relationship between *Pseudobiceros* and *Thysanozoon* is supported though by both genera possessing two male pores and the same pharynx structure. When *Pseudobiceros* was constrained (MP analysis) into a monophyletic clade, the resulting tree was not significantly longer (2 additional steps, α=0.05) as determined by the non-parametric ranked sign test of Templeton (Larson, 1994). More importantly though, constraining a monophyletic *Pseudobiceros* did not influence the topology of relationships within *Pseudoceros* (Fig. 2). As a consequence, *Pseudobiceros* should be considered a valid genus, distinct from *Pseudoceros*, *Thysanozoon* or *Phrikoceros.* Additionally, we recommend that alternative trees that are supported by morphological characters be favoured, as long as they are not statistically different from the MP trees. The genetic distance between the two specimens of *P. uniarborensis* was 1.25%, a value that was comparable to the intraspecific distance of *Pseudoceros bifurcus.*

An examination of intergeneric distances (Table 3), as expected, resulted in larger values than values within a genus. For example, genetic distances between *Acanthozoon* and *Tytthosoceros, Maiazoon,* and the average of the two *Thysanozoon* specimens was 5.584%, 2.052% and 4.997%%, respectively (Table 3). These values were comparable to values common for other flatworms (Litvaitis et al., 1994; Litvaitis & Rohde, 1999). Not surprisingly, the closest intergeneric distance was found between *Maiazoon* and *Pseudobiceros* (2.048%). The two genera are both characterized by two male reproductive systems, the same type of simple pharyngeal folds, and a similar marginal ruffling (Table 1). However, characters of the female reproductive system clearly separate them into two genera (Newman & Cannon, 1996).

Comparisons of genetic distances for *Phrikoceros* and *Maiazoon* with the remaining genera examined in this study, revealed their distinctness (Table 3). *Phrikoceros*, which shares characters with Pseudoceros and Pseudobiceros, was more closely related to *Pseudobiceros* (2.354%); however, this value was still higher than the intrageneric values determined for *Pseudobiceros.*

Using a morphology independent data set, the present study confirmed and validated the genera *Pseudoceros* and *Pseudobiceros* as taxonomic entities. Additionally, we were able to confirm the use of colour patterns to distinguish species. Species identifications based on nucleotide sequence although unequivocal, result in the destruction of the specimen. Thus, the colour scheme of Newman & Cannon (1994a, 1995)

which preserves specimens intact, is a significant and valid contribution to polyclad systematics. Finally, the present study also supported the establishment of *Phrikoceros, Maiazoon* and *Tytthosoceros* as separate genera (Newman & Cannon, 1996) and their relationships to other genera within the family Pseudocerotidae.

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